ANTI-OPISTHORCHIASIS AND HEPATOPROTECTIVE EFFECTS OF *POPULUS TREMULA* BARK EXTRACT: *IN VITRO* STUDIES

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The problem of opisthorchiasis caused by *Opisthorchis felineus* requires a search for alternative treatments, because the trematode can develop drug resistance and the existing drugs, such as praziquantel, may have undesirable side effects. This study aimed to identify the anti- opisthorchiasis and hepatoprotective properties of *Populus tremula L.* bark extract using *in vitro* methods. The PGF-PT active fraction with a high content of phenol glycosides was obtained though extraction. PGF-PT showed a dose-dependent anti-opisthorchiasis effect: the relative mobility index of maritae decreased from 98.2% at 250 μg/ml to 54.5% at 2000 μg/ml ($p < 0.05$), and the proportion of immobile specimens at 2000 μg/ml was 30% ($p < 0.05$). The probable mechanism of action is focal damage to the cells of the parasite's superficial epithelium (tegument). PGF-PT exhibited a pronounced hepatoprotective effect in a lipotoxicity model based on HepG2 cells, as evidenced by normalized intracellular lipid accumulation and reduced oxidative stress. A high antioxidant activity of PGF-PT was shown in the model system (IC_{50} = 79.3 ± 1.0 μg/ml). Thus, the PGF-PT fraction has a complex effect — eliminating parasites and correcting metabolic disorders in the liver — which makes it a promising basis for new effective anthelmintic drugs.

Keywords: *Opisthorchis felineus*, *Populus tremula* bark extract, anthelmintic therapy, praziquantel, phenol glycosides, antioxidants, tegument

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Compliance with ethical standards: the study was approved by the Institutional Animal Care and Use Committee of the Preclinical Research Center, Central Research Laboratory, Siberian State Medical University of the Ministry of Health of the Russian Federation (Resolution No. 1 of September 02, 2023). The work was performed in full compliance with current drug circulation regulations, guidelines for the use of laboratory (experimental) animals in preclinical (non-clinical) studies, and sanitary and epidemiological standards.

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ПРОТИВООПИСТОРХОЗНАЯ И ГЕПАТОПРОТЕКТОРНАЯ АКТИВНОСТЬ ЭКСТРАКТА КОРЫ ОСИНЫ (*POPULUS TREMULA L.*) В ЭКСПЕРИМЕНТАХ *IN VITRO*

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Для решения проблемы описторхоза, вызванного трематодой *Opisthorchis felineus*, необходим поиск альтернативных терапевтических решений ввиду рисков развития лекарственной устойчивости и возможных побочных эффектов существующих препаратов, таких как празиквантел. Цель исследования — выявить противоописторхозные и гепатопротекторные свойства экстракта коры осины (*Populus tremula* L.) на примере *in vitro*-моделей. Методом экстракции выделена активная фракция PGF-PT с высоким содержанием фенолгликозидов. Выявлен дозозависимый антигельминтный эффект PGF-PT: индекс относительной подвижности марит снизился с 98,2% (250 мкг/мл) до 54,5% (2000 мкг/мл, $p < 0,05$), а доля неподвижных особей при концентрации 2000 мкг/мл достигла 30% ($p < 0,05$). Вероятный механизм действия — индуцированное очаговое повреждение клеток поверхностного эпителия (тегумента) паразита. Выявлен выраженный гепатопротекторный эффект PGF-PT, подтвержденный моделью липотоксичности на клетках HepG2: наблюдались нормализация накопления внутриклеточных липидов и подавление окислительного стресса. В модельной системе показана высокая антиоксидантная активность PGF-PT (IC_{50} = 79,3 ± 1,0 мкг/мл). Таким образом, фракция PGF-PT обладает комплексным действием, направленным на элиминацию паразита и коррекцию метаболических нарушений в печени, что открывает перспективы для создания на ее основе новых эффективных препаратов для антигельминтной терапии.

Ключевые слова: *Opisthorchis felineus*, экстракт коры осины, антигельминтная терапия, празиквантел, фенолгликозиды, антиоксиданты, тегумент

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Соблюдение этических стандартов: исследование одобрено комиссией по контролю содержания и использования лабораторных животных центра доклинических исследований центральной научно-исследовательской лаборатории (ЦДИ ЦНИЛ) ФГБОУ ВО СибГМУ Минздрава России (протокол № 1 от 2 сентября 2023 г.). Работа выполнена в полном соответствии с действующими нормативно-правовыми документами об обращении лекарственных средств, рекомендациями по работе с лабораторными (экспериментальными) животными при проведении доклинических (неклинических) исследований, соблюдением санитарно-эпидемиологических норм.

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The problem of opisthorchiasis caused by the trematodes *Opisthorchis felineus* and *Opisthorchis viverrini* remains extremely urgent in modern medicine, parasitology, and epidemiology [1]. The endemic regions extend across parts of the Russian Federation (Western and Eastern Siberia, Cis-Urals), Eastern Europe, Kazakhstan, and Southeast Asian countries (Thailand, Laos, Vietnam) [2]. According to WHO, more than 80 million people in the world are at risk, and the number of infected is estimated at several tens of millions [3]. The only drug used for etiotropic therapy is praziquantel (PZQ); however, its widespread use poses a potential risk of generating resistant parasite strains, which could render the medicine completely ineffective [4]. In addition, several recent reports suggest that repeated courses of PZQ may increase the risk of cholangiocarcinoma, likely due to the massive release of parasitic antigens associated with the treatment [4, 5]. Therefore, the search for alternative medicines that are both effective and low in toxicity has become particularly urgent.

Extracts derived from parts of trees in the Populus family, particularly their buds and bark, are traditionally used due to their anti-inflammatory, antioxidant, and hepatoprotective properties confirmed by studies [6, 7]. Since oxidative stress and chronic inflammation play key roles in the development of opisthorchiasis complications, and plant extracts with similar structures — such as artesunate — have already proven effective in treating other trematodes [8], the bark extract of *Populus tremul*a L. (*P. tremula*) appears to be a promising subject for further study. The pharmacological activity of aspen (*P. tremula* L.) bark extracts mainly stems from the high content of phenol glycosides of the salicinoid class [9]. Studies have shown that these phytochemical components have a pronounced antiparasitic effect against a wide range of etiological agents causing trematodiasis (e.g., *Opisthorchis spp.*, *Psilostomum*) and helminthiasis (including nematodosis and protozoal infections such as giardiasis) [10, 11].

The purpose of this study was to investigate the properties of *P. tremula* bark extract that is rich in phenol glycosides.

METHODS

The object of the study was the *P. tremula* bark extract with a high content of phenol glycosides.

At the facilities of Biolit, a research and production company, we made a liquid extract with the feedstock to product ratio of 1:1. The feedstock was the bark of *P. tremula* harvested in autumn and winter, naturally dried to a residual humidity of 8–10%; the extraction was performed in a 2000 L industrial extractor (Hubei weihua machinery co., ltd, China) using the repercolation method. The extractant was water; the resulting liquid extract was condensed in an SX-250 vacuum evaporation system (Hubei weihua machinery co., ltd, China) to a residual moisture content of 25–40%.

The phenol glycoside–rich fraction (PGF-PT) was prepared in the Quality Control Laboratory of the Central Research Laboratory, Siberian State Medical University, as follows: the thick extract was reconstituted with purified water at a ratio of 1:15, mixed for 30 minutes, and enriched with a threefold (v/v) excess of 96% ethanol. After allowing the sediment to settle at 4°C for 12 hours, the supernatant was decanted and centrifuged at 4000 rpm for 3 minutes (Eppendorf 5702, Eppendorf, USA). The decanted solution was combined with the supernatant and concentrated to one-tenth of its original volume using a Rotavapor V-3 rotary evaporator (Buchi, Switzerland) equipped with a PC 500 vacuum pumping station (Vacuubrand, Germany) at a water bath temperature of 40 °C. The concentrated solution was then dried by convection. The yield of PGF-PT was 71.65 ± 13.4%. Using HPLC, we standardized PGF-PT by quantifying its salicin phenol glycoside content in an Ultimate 3000 chromatograph (Dionex, Thermo, Germany) equipped with a DAD3000 diode matrix detector on a Zorbax Eclipse plus C18 3.0 × 150 mm, 3.5 µm column, mobile phase A — 0.1% trifluoroacetic acid in water, mobile phase B — acetonitrile. The elution was gradient, the volume of the introduced sample was 20 µl, and the temperature of the column thermostat — 30 °C. We identified the salicin peak by spectral features: absorption maxima at wavelengths of 194 and 269 nm, and the shoulder at a wavelength of 215 nm. The salicin content was determined by the external standard method. The salicin peak retention time was 7.544 minutes. Tannin content, representing polyphenolic compounds, was quantified permanganometrically as specified in applicable regulations; it equaled 8.16 ± 0.41%, and the content of salicin was 4.7 ± 0.6%.

The anti-opisthorchiasis activity of PGF-PT was evaluated at the Preclinical Research Center of the Central Research Laboratory using maritae of the hepatic fluke *O. felineus*. Praziquantel (PZQ, TCI, Japan) at a dose of 600 µg/ml was used as a comparison drug [11]. The experimental animals were infected using the described method [12]. A total of 40 specific pathogen-free (SPF) female golden hamsters (*Mesocricetus auratus*), weighing 130–150 g, were used in the experiment. The animals were obtained from the Federal Research Center, Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences. Three months after infection, the animals were euthanized in a CO_2 atmosphere. Maritae were extracted from the hepatobiliary tract, washed with 0.9% NaCl solution, and incubated at 37 °C in a 5% CO_2 atmosphere for 2 hours in RPMI 1640 culture medium containing 2× penicillin–streptomycin [13]. The maritae were placed in a six-well culture plate, ten specimens per well, containing RPMI 1640 medium supplemented with 1× penicillin-streptomycin, 10 g/L glucose, and 2 g/L sodium bicarbonate.

Solutions of the PGF-PT and PZQ fractions were prepared in dimethyl sulfoxide (DMSO), followed by dilution in an incubation medium. The studied PGF-PT fraction was tested at final concentrations of 250, 500, 1000, and 2000 µg/ml. As a negative control, we used samples incubated in a medium containing a similar volume of DMSO (up to 0.5%) to exclude the possible nonspecific effects of the solvent itself on the metabolism and behavior of the maritae. The incubation of maritae with the substances lasted 24 hours in a CO_2 incubator.

The efficacy of anthelmintic drugs is tested *in vitro*, by assessing the mobility and morphology of parasites [10, 11]. Thus, we assessed the antiparasitic activity of the studied agent by analyzing changes in the mobility and tegument morphology of the maritae using a NE910 microscope (Ningbo Yongxin Optics Co., Ltd., China) equipped with a Digital Sight 1000 camera (Nikon, China). Mobility was assessed visually using a 4-point scale [14]: 4 — very active movements, similar to those of control flukes; 3 — regularly active movements, less energetic than the control, but involving the entire body; 2 — low activity, with limited movement; 1 — immobile worms. The mobility indicators measured in the wells were normalized to the values of the control group.

The relative mobility index (RM) was calculated by the following formulas [14]:

$$RM\ \% = \frac{MI\ experiment}{MI\ control}$$

$$MI = \frac{\Sigma n \times Nn}{\Sigma Nn},$$

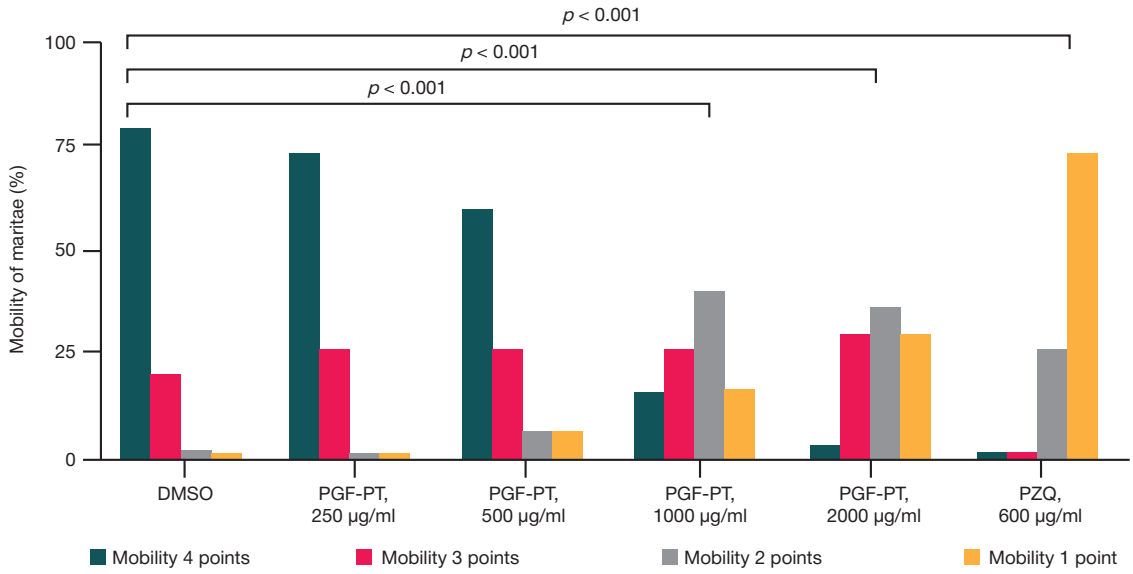

Fig. 1. The effect of the PGF-PT fraction on the mobility of *O. felineus* maritae

where *MI* — the index of mobility of the worm group, *n* — mobility score (points), Nn — the number of trematodes given n points.

To determine the effect of the extract on the functional state of the liver fluke's tegument, we studied the specimens using propidium iodide (PI), a fluorescent intercalating dye that binds to DNA and stains the nuclei of dead cells. The maritae were incubated with PI (2 μg/ml) for 20 minutes, then washed with saline solution. The images were made with a Leica DM6 fluorescence microscope (Leica Microsystems GmbH, Germany) [15].

The hepatoprotective activity of PGF-PT was evaluated in vitro using a lipotoxicity model induced by a mixture of oleate and sodium palmitate in HepG2 hepatocellular carcinoma cells, by measuring intracellular lipid levels and reactive oxygen species (ROS). The lipid content was assessed after staining with Nile red fluorescent dye [16]. For positive control, we used 100 μM of gemfibrozil, a lipid-lowering compound (Sigma-Aldrich, USA). The lipid droplets were visualized using a Leica DM6 fluorescence microscope (Leica Microsystems GmbH, Germany). The level of ROS in cells was measured using 2.7'-dichloro-dihydro-fluorescein diacetate (DCFH-DA) [17]. The safe concentrations of PGF-PT for use in this model (31.25, 62.5, and 125 μg/ml) were selected by testing with a neutral red vital dye [18].

To evaluate the degree of contribution of the direct anti-radical effect to the fraction's ability to influence ROS levels in cells, we set up an experiment in the model system that involved a stable radical of 1.1-diphenyl-2-picrylhydrazyl (DPPH), as described previously [19]. The activity was expressed as the IC50 index, which reflects the effective concentration of PGF-PT at which 50% of the free radicals of DPPH are reduced.

Statistical data analysis was performed in GraphPad Prism 8.0 (GraphPad Software, USA). Pearson's chi-square test (χ^2) was used to analyze the ordered data. For pairwise group comparisons, we applied the Bonferroni correction. The normality of the distribution of the quantitative variables was assessed using the Shapiro–Wilk test. The mean values of several independent groups were compared using one-way analysis of variance (ANOVA). The threshold of statistical significance was $p < 0.05$.

RESULTS

The PGF-PT fraction had a dose-dependent effect on the mobility of *O. felineus* maritae (Fig. 1).

In the control group, the maritae maintained their mobility and integrity of the tegument. Treatment with the PGF-PT fraction at a concentration of 1000 μg/ml reduced the mobility of 40% of adult worms to a minimum value (2 points on the mobility scale), and 17% of the parasites became completely motionless (1 point on the mobility scale). An increased concentration of 2000 μg/ml increased the proportion of motionless specimens to 30%. PZQ, the comparison drug, completely immobilized 73% of maritae at a concentration of 600 μg/ml.

In the control group, the parasite mobility index was 3.8. As the concentration of the PGF-PT fraction increased from 250 to 2000 μg/ml, the index decreased from 3.7 to 2.1, and the relative mobility of the worms dropped from 98.2% to 54.5%. PZQ, in turn, reduced parasite activity to 28.5%, inducing spastic paralysis (Table).

Examination of tegument integrity in the control group showed that the PI fluorescent dye failed to penetrate the cells of the maritae. After treatment with PZQ, the fluorescent glow localized in the areas of tegument syncytium. These results are consistent with the published studies that report PZQ's capability to induce peeling of tegument and destruction of *O. viverrini* [15]. Application of PGF-PT at concentrations of 500, 1000, and 2000 μg/ml allowed PI to partially penetrate deep into the tegument syncytium in certain areas of the worm's body, indicating local cell membrane disruptions and breach of tissue integrity (Fig. 2).

Table. The effect of PGF-PT on the mobility index (MI) and relative mobility index (RM) of *O. felineus* maritae

	DMSO	PGF-PT, 250 μg/ml	PGF-PT, 500 μg/ml	PGF-PT, 1000 μg/ml	PGF-PT, 2000 μg/ml	PZQ, 600 μg/ml
MI	3.8	3.7	3.4	2.4	2.1	1.1
RM, %		98.2	89.5	64	54.5	28.5

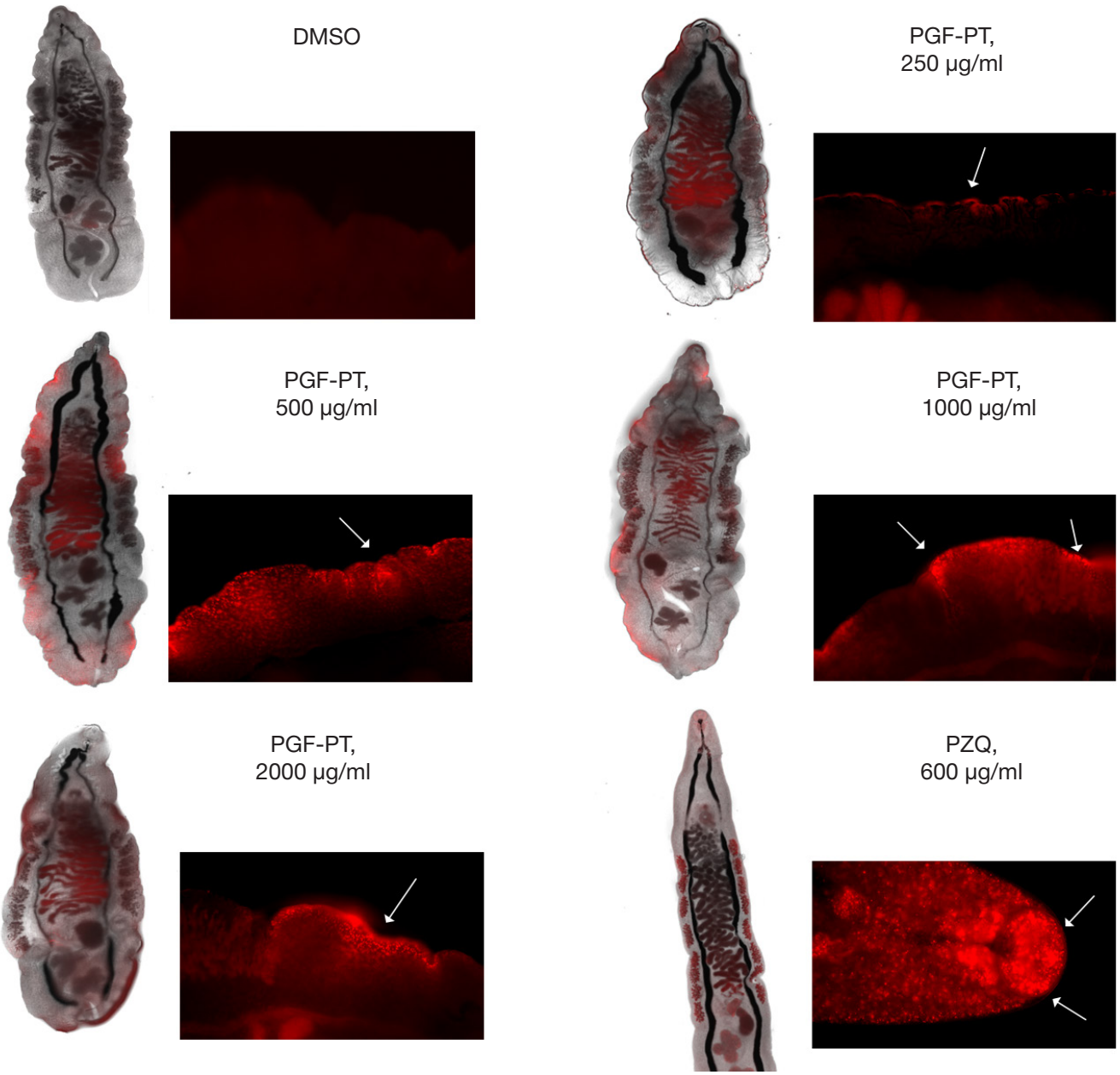

Fig. 2. Assessment of damage to the *O. felineus* maritae tegument by fluorescent staining

Thus, exposure to PGF-PT caused partial degradation of the tegument cells but did bring complete functional destruction of the parasite's organism and loss of mobility in all the cases considered.

Light microscopy confirmed significant damage to the surface structures of the maritae's bodies after incubation with high concentrations of PGF-PT (1000 and 2000 μg/ml): massive peeling of the epithelium and edema of the tegument (see appendix).

Incubation of HepG2 cells in a medium with oleic and palmitic fatty acids (FA) induced hepatotoxicity. According to the assessment of changes in the fluorescence intensity of the lipophilic dye (Fig. 3A), which was consistent with microscopy data (Fig. 3B), the intracellular lipid content increased by 55% ($p < 0.05$). The fluorescence intensity of DCFH-DA also increased by 57% ($p < 0.05$), following the growth of the level of ROS in the cells (Fig. 3B).

At concentrations of 62.5 and 125 μg/ml, PGF-PT pushed the lipid levels down by 32.9% ($p < 0.05$) and 48.9% ($p < 0.05$), respectively; this effect was comparable to that of gemfibrozil, a lipid-lowering compound ($p < 0.05$) (Fig. 3A). Meanwhile, the PGF-PT fraction reduced oxidative stress in FA-treated HepG2 cells (Fig. 3B). The introduction of PGF-PT at concentrations of 31.25, 62.50, and 125.00 μg/ml reduced the intensity of fluorescence of DCFH-DA ROS probe by 12.6% ($p < 0.05$), 28.2% ($p < 0.05$) and 36.2% ($p < 0.05$), respectively.

The decrease of intracellular ROS levels may also result from the PGF-PT's direct anti-radical properties, the assessment of which in the model system is shown in Figure 4A.

Based on the data obtained, we calculated the concentration of the PGF-PT fraction that ensures inhibition of 50% of DPPH free radicals (IC50): 79.3 ± 1.0 μg/ml (Fig. 4B). Thus, the results of the study indicate the pronounced anti-radical and hepatoprotective properties of the PGF-PT fraction.

DISCUSSION

This study demonstrates pronounced anti-opisthorchiasis and hepatoprotective potentials of the *P. tremula* bark extract with high content of phenol glycosides (PGF-PT fraction).

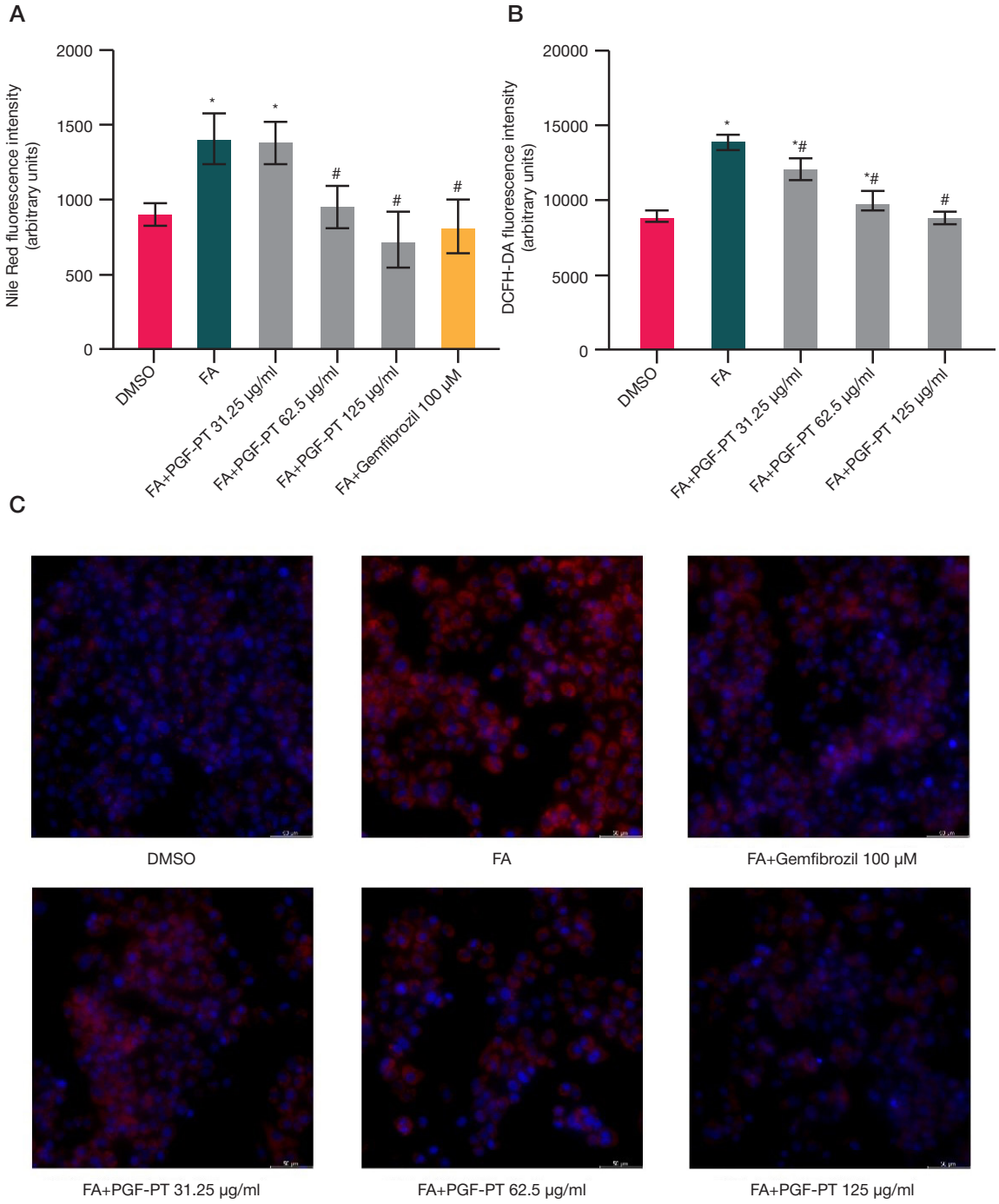

Fig. 3. Hepatoprotective activity of PGF-PT, estimated by the level of intracellular lipids and reactive oxygen species (ROS) in HepG2 cells after incubation in a medium containing oleic (1 mM) and palmitic (0.5 mM) fatty acids (FA): **A** — intensity of Nile red fluorescence (intracellular lipids), **B** — intensity of 2,7'-dichlorodihydrofluorescein diacetate fluorescence (ROS), and **C** — fluorescence images of cells (magnification x40) stained with Nile red and Hoechst 33342 (cell nuclei). The data is presented as M ± SD. * — the differences from the DMSO group are significant ($p < 0.05$); # — the differences from the FA group are significant ($p < 0.05$))

We found that this fraction exhibited a dose-dependent ability to reduce the mobility of *O. felineus* maritae. At the highest tested concentration (2000 µg/ml), 30% of the worms became completely motionless. Although this effect was lower than that achieved with PZQ (73% of specimens motionless), it indicates that developing a drug based on PGF-PT is a promising direction.

Microscopic examination confirmed that exposure to PGF-PT causes partial degradation of the tegument cells, which limits the functional capabilities of the parasite. However, the destruction was incomplete, and the parasite retained the ability to move to a certain degree, which underscores the need to continue research to enhance the anthelmintic effect. Tegument antigens from this multinucleated syncytium, approximately 4 µm thick and performing key functions like immune evasion, are promising for anthelmintic drug or vaccine development. Interestingly, anthelmintic drugs with different mechanisms of action cause damage to the parasite tegument [11]. This indicates that the drugs, along with their direct effect on molecular targets, disrupt the surface of the parasite, which activates the antibody-dependent cellular cytotoxicity and provokes an effective immune response of the host organism.

These data on the dose-dependent suppression of *O. felineus* mobility and focal tegumental damage align with prior reports of strong antiparasitic effects from phenol glycosides in *Populus spp*. Recent studies describe the anti-opisthorchiasis effect of crude

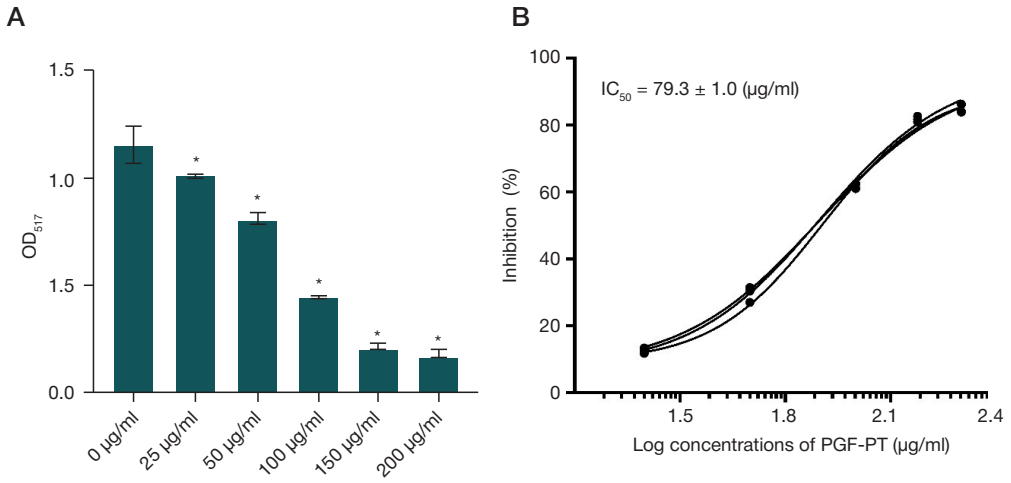

Fig. 4. The effect of the PGF-PT fraction on the optical density of a 2,2-diphenyl-1-picrylhydrazyl solution (DPPH, 100 µm) (**A**) and the semi-logarithmic dependence of the DPPH radical reaction inhibition effectiveness and the concentration of the PGF-PT fraction (**B**). The data is presented as M ± SD. * — the differences from the control group (0 µg/ml) are significant ($p < 0.05$)

extracts of *Allium sativum* L. [11] and Thunbergia laurifolia Lindl. [20]. Other works investigate the anthelmintic properties of flavonoid quercetin, a polyphenolic compound; it is capable of suppressing mobility of the parasites and damaging their tegument [21, 22]. A milder mechanism of action may help reduce the risk of severe immunopathological reactions associated with mass death and destruction of parasites.

The hepatoprotective effect of PGF-PT — manifested as decreased lipotoxicity and oxidative stress in the HepG2 cell model — is supported by current data on the properties of phenol glycosides and other bioflavonoids. The hepatoprotective effect of polyphenols was shown to rely on a complex of activities, including enhancement of antioxidant action, metabolism, and immunomodulation [23]. This suggests that therapy with this extract not only reduces the parasitic load, but also protects the liver from damage, which is especially important in chronic forms of opisthorchiasis and in view of the risk of cholangiocarcinoma.

CONCLUSIONS

The PGF-PT fraction from the *Populus tremula* bark shows a pronounced anthelmintic activity against *Opisthorchis felineus in vitro*. The effect is dose-dependent; the fraction suppresses mobility of the parasite and damages its tegument. In addition, PGF-PT protects the liver, reducing lipotoxicity and oxidative stress in hepatocytes, and, as opposed to praziquantel, offers a lower risk of immunopathological complications due to milder action. These data highlight the potential of PGF-PT as a promising component of new anti-opisthorchiasis drugs with additional hepatoprotective effect. *In vitro* systems have significant limitations in terms of reproduction of a real multicomponent "parasite-host" system, but they can give important information about the basic mechanisms of pharmacological action. The results of this study serve as the basis for further *in vivo* investigation aimed at evaluating the efficacy and safety of the PGF-PT fraction in real biological conditions.

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